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CHROMOSOMAL CHARACTERISTICS OF NORTH AMERICAN AND OTHER NAIADES (BIVALVIA: UNIONIDA)

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ABSTRACT

This project was conducted to determine if chromosomal characteristics can provide in-formation useful in systematics of the naiades (Bivalvia: Unionida). A field-compatible slide preparation technique was perfected to the point that a few chromosome spreads could be expected from most processed animals. Chromosome slides were prepared from 250 naia-des collected in ten of the east-central and southeastern United States, the western state of Oregon, and Manitoba Province, Canada.
The 65 species of North American naiades for which chromosome numbers were determined during this project (64 members of the family Unionidae and one member о the family Marga-ritiferidae) allhave a diploid number of 38 chromosomes. This number is identical to reported diploid numbers for the 11 European and seven of the eight Asian unionid and margaritiferid species that have been studied, strongly suggesting that 38 is the diploid number present in the largely Northern Hemisphere superfamily Unionoidea. In the other unionid superfamily, the Etherioidea, the three Australian and one South American hyriid naiades that have been studied all have 34 diploid chromosomes, while the single studied African iridinid species has 20 chromosomes. If this difference in chromosome numbers is substantiated as more, especially Southern Hemisphere species are studied, this straight-forward morphologic character could become important in recognizing distinctions between high-level groups of naiades.
An analysis of chromosomal morphology focused on two metrics: Arm Ratio (r) and Rela-tive Length (RL). When considered together, these two virtually independent metrics sort the chromosomes into similar-size and similar-shape groups that can facilitate comparisons between species. Analysis ofthe suggested karyotypes for these two metrics and the detailed karyotypes of their interaction demonstrates the similarity of two species in the same naiad genus and the substantial dissimilarity of two species in different naiad tribes. Analysis of the
r and RL metrics for ten species and an all-inclusive composite demonstrates that karyotypic data can recognize similarities and differences among naiad species that correspond with results from other morphologic and genetic character sets.
Key words: Bivalvia, Unionida, naiad, chromosome, karyotype, relative length.

INTRODUCTION

All of the approximately 900 species of large
bivalve mollusks that occur in freshwater habi-
tats around the world are considered members
of the order Unionida (Graf & Cummings,
2006). A variety of English common names have been used when considering all of these species as a group (freshwater mussels, clams, unionids, etc.); | prefer to use “naiad,” (plural: naiades) because - as it is used by Simpson (1896), Ortmann (1921), and others — naiad
is the only broadly inclusive term for all of the members of this order that is not burdened by any taxonomic bias or inaccuracy. Fos-sil evidence suggests that the naiades have

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existed in freshwater habitats since Paleozoic time (Giribet, 2008). The combination of their large size, often obvious presence in freshwa-ter habitats, world-wide distribution, and long geologic history have made the naiades inter-esting to a long list of naturalists, malacologists and evolutionary biologists (e.g., Say, 1817; Baker, 1928; Fuller, 1974; Haag, 2012). Over the years, researchers have used a variety of shell, anatomical, developmental and genetic characteristics to suggest evolutionary and taxonomic relationships among various groups of naiades (e.g., Lea, 1870; Simpson, 1896; Ortmann, 1921; Davis & Fuller, 1981; Graf & Cummings, 2007). To date, however, no au-thors have used counts of the chromosomes

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or other chromosomal characteristics in their studies of naiad relationships.
Apparently, Lillie (1901) was the first to
publish a count of the chromosomes present
in any naiad. In his extensive study of naiad
eggs and their development, Lillie saw many sectioned chromosome spreads and reported that Unio complanata (= Elliptio complanatus) has 16 haploid [= 32 diploid] chromosomes (Lillie, 1901). Both Menzel (1968) and Patter-son (1969) found it difficult to make accurate counts of the chromosomes from sectioned material and chose not to include counts based on sectioned material in their lists of molluscan chromosome numbers. That precedent is fol-lowed in this paper.
In their monograph on naiades in Austra-lia, McMichael & Hiscock (1958) included a paragraph indicating that Alathyria pertexta, Velesunio ambiguus and Velesunio legrandi all have 34 diploid chromosomes. Patterson (1969) tabulated many previous reports of mollusk chromosome numbers, listing 1,100 chromosome counts for species of gastropods, two for cephalopods, and 22 for pelecypods. None of the bivalve chromosome numbers cited by Patterson were for naiades. Van Griethuysen et al. (1969) described 38 diploid chromosomes for two European naiad species, and Nadamitsu & Kanai (1975, 1978) reported 38 diploid chromosomes for three Japanese naiades. A preliminary report on this project (Jenkinson, 1976) indicated that 15 North American naiad species all have 38 diploid chromosomes.
In more recent years, Nakamura (1985) accumulated published chromosome data on the molluscan classes Bivalvia, Polypla-cophora and Cephalopoda. His list includes chromosome numbers for 125 bivalve spe-cies, including seven naiad species beyond the 15 reported by Jenkinson (1976). Park & Burch (1995) reported chromosome numbers for two additional North American naiades and recognized chromosome numbers for seven Korean species studied by Park & Kwon (1991). Thiriot-Quiévreux published compila-tions of recent chromosome counts for aquatic organisms (Thiriot-Quiévreux, 1994), bivalves (Thiriot-Quievreux, 2002) and gastropods (Thiriot-Quievreux, 2003). By 2002, her bivalve list included 66 species, four of which were naiades: two from Europe, one from Africa and one from China (Thiriot-Quiévreux, 2002). In their review of bivalve chromosomal banding studies, Leitao & Chaves (2008) listed chro-

mosome numbers for 48 additional species, including two naiades: one from Europe and one from Asia. Table 1 presents a summary of published chromosome numbers for the order Unionida, aggrading records from a variety of Sources.
The pattern indicated early on by the exten-sive work on gastropods and more sparse work on the other classes suggested that mollusks show a remarkable degree of conservation in chromosome number (Patterson, 1969; Murray, 1975). Based on more extensive and more recent data, Thiriot-Quiévreux (2002: 197) observed that: “This pattern remains ... but this review shows that, as more species are examined, the range of chromosome numbers increases.” Information on chromosome num-bers accumulated by various authors (many tabulated in Thiriot-Quiévreux, 2002; and Leitäo & Chaves, 2008) indicates that bivalve diploid numbers range between 12 and 213, with complete uniformity in the Osterida (all 20), an extremely wide range in the Venerida (from 20 to 213), and more modest ranges in the other orders. The most common diploid chromosome number found within the Bivalvia IS 38, present in 40% of the species examined So far. As indicated in Table 1, 38 is the typi-cal chromosome number within the Unionida. The only exceptions reported so far are three species from Australia (all 34), two from Egypt (one 20 and one 28), one from Chile (34), and one from Thailand (37).
This project was conducted to determine if chromosomal characteristics of North Ameri-can naiades could be examined as a routine procedure and if the study of these character-istics could provide information useful in naiad systematics. Much of the original research presented here is taken from a dissertation | completed and defended in 1983 (Jenkinson, 1983). At that time, | was deeply involved in the first of a series of large projects for my post-graduate employer and, although an oral summary of this work was presented at the 1983 American Malacological Union meeting (Jenkinson, 1984), the body of this work has remained unpublished until now. Recently, it has come to my attention that the few people who remember this project believe it demon-strated that chromosomal characteristics would not help clarify evolutionary and taxonomic relationships among the naiades. The work | conducted 30 years ago convinced me ofj ust the opposite; it showed me that chromosomal characteristics offer a new perspective on how

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TABLE 1. Published chromosome numbers for the Order Unionida. Some of these entries are included in the reviews by Nakamura (1985), Park & Burch (1995), Thiriot-Quiévreux (2002) and Leitäo & Chaves (2008). The suprageneric groupings follow Graf & Cummings (2007). Most author and date entries come from the cited references; others come from Graf & Cummings (2007) and Simpson (1900).

Diploid
Species No. Location References

Superfamily Etherioidea
Family Hyriidae
Alathyria pertexta lredale, 1934 34 Е Australia McMichael & Hiscock, 1958
Diplodon chilensis (Gray, 1828) 34 Villarrica Lake, Chile Jara-Seguel et al., 2000
Velesunio ambiguus (Philippi, 1847) 34 SE Australia McMichael & Hiscock, 1958
Velesunio legrandi (Petterd, 1889) 34 Tazmania McMichael & Hiscock, 1958

Family Iridinidae
Mutela rostrata (Rang, 1835) 20 Egypt Ebied, 1998

Superfamily Unionioidea
Family Margaritiferidae
Margaritifera laevis (Hass, 1910) 38 Hiroshima Prefecture, Nadamitsu & Kanai, 1975
Japan
Margaritifera margaritifera form falcata 38 Willamette River, Jenkinson, 1976
(Gould, 1850) Oregon
Family Unionidae

Africa
Unio elongatulus Pfeiffer, 1825 20 Egypt Ebied, 1998

Asia
Anodonta arcaeformis flavotincta 38 Korea Park & Kwon, 1991
von Martens, 1905
Anodonta woodiana (Lea, 1834) 38 Korea Park et al., 1988; Park &

Anodonta woodiana elliptica Heude, 1878 38 China
Anodonta woodiana pacifica 38 China
Heude, 1878
Cristaria plicata (Leach,1815) 38 China
Hyriopsis cumingii (Lea, 1852) 38 China
Inversidens japonensis (Lea, 1859) 38 Iwakuni, Japan
Lamprotula gottschei 38 Korea
(von Martens, 1894)
Lanceolaria acrorrhyncha 38 Korea
(von Martens, 1894)
Pseudodon obovalis omiensis 38 Miyoshi, Japan
(von Heimburg, 1884)
Solenia khwaegnoiensis "37" Khwae Noi River,
Panha & Deein, 2003 Thailand
Unio douglasiae 38 Korea
(Griffith & Pidgeon, 1834)

Kwon, 1991
Shi, 1980
Wen et al., 2009

Shi, 1980
Shi, 1980; Wang et al., 2000
Nadamitsu & Kanai, 1978
Park & Kwon, 1991

Park & Kwon, 1991

Nadamitsu & Kanai, 1978

Deein et al., 2003

Park & Kwon, 1991

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Species

Unio douglasiae sinuolatus
von Martens, 1905
Sorenia triangularis (Heude, 1885)

Europe
Anodonta anatina (Linnaeus, 1758)

Anodonta cygnea (Linnaeus, 1758)

Anodonta stagnalis
(Gmelin in Linnaeus, 1758)
Anodonta woodiana (Lea, 1834) Anodonta zellensis Model, 1945

Colletopterum piscinale (Drouet, 1881)

Colletopterum ponderosum
Bourguignat, 1880
Pseudanodonta complanata
(Rossmaessler, 1835)
Unio conus Spengler, 1793
Unio elongatulus Pfeiffer, 1825
Unio pictorum (Linnaeus, 1758)

North America
Alasmidonta arcula (Lea, 1838) Alasmidonta marginata Say, 1818 Anodontoides ferussacianus (Lea, 1834)

Elliptio complanata (Lightfoot, 1786) Epioblasma triquetra (Rafinesque, 1820) Gonidea angulata (Lea, 1838)
Lampsilis radiata luteola (Lamarck, 1819) Lasmigona costata (Rafinesque, 1820) Potamilus alatus (Say, 1817)
Ptychobranchus fasciolaris
(Rafinesque, 1820)
Pyganodon [as Anodonta] grandis (Say, 1829)
Quadrula quadrula (Rafinesque, 1820) Toxolasma lividus Rafinesque, 1831 Tritogonia verrucosa (Rafinesque, 1820) Villosa iris iris (Lea, 1829)
Villosa lienosa (Conrad, 1834)

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Diploid
No. Location References

38 Korea Park & Kwon, 1991

38 Korea Park & Kwon, 1991

38 Damien Lake, Nether- van Griethuysen et al.,

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lands; Wulpinskie 1969; Woznicki & Jankun,
Lake, Poland 2004
Lagoa de Mira, Portu- Carrilho et al., 2008; Melny-
gal; Ukraine chenko & Yanovich, 2004
Ukraine Melnychenko & Yanovich,
2004
Konin Lakes, Poland Woznicki, 2004
Ukraine Melnychenko & Yanovich,
2004
Ukraine Melnychenko & Yanovich,
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Ukraine Melnychenko & Yanovich,
2004
Ukraine Melnychenko & Yanovich,
| 2004
Ukraine Melnychenko, 2000
Brenta River, Italy Vitturi et al., 1982
Damien Lake, Nether- van Griethuysen et al.,
lands; Ukraine 1969; Melnychenko, 2000

Ohoopee River, Georgia Jenkinson, 1976
Big Darby Creek, Ohio Jenkinson, 1976
Buffalo Creek, Ohio; Jenkinson, 1976; Park &
Michigan Burch, 1995
Michigan Park & Burch, 1995
Michigan Park & Burch, 1995
Willamette River, Oregon Jenkinson, 1976
Big Darby Creek, Ohio Jenkinson, 1976
Big Darby Creek, Ohio Jenkinson, 1976
Buck Creek, Kentucky Jenkinson, 1976
Big Darby Creek, Ohio; Jenkinson, 1976; Park &
Michigan Burch, 1995
Michigan, Colorado; Park & Burch, 1995; Jen-
Buffalo Creek, Ohio kinson, 1976
Big Darby Creek, Ohio Jenkinson, 1976
Buck Creek, Kentucky Jenkinson, 1976
Big Darby Creek, Ohio Jenkinson, 1976
Big Darby Creek, Ohio Jenkinson, 1976
Buffalo Creek, Ohio Jenkinson, 1976

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closely various groups of naiades are, and are not, related to each other.
When | started to consider publishing this research, | had no desire to reexamine all of the slides and chromosome spreads, or to re-analyze all of the data that had been generated thirty years ago. | did, however, recognize the need to update the identifications of the species that had been included in my work. All of the naiades and other bivalves examined as part of this project had been deposited at the Ohio State University Museum of Biological Diver-sity (OSUM) and, over the years, had been catalogued into that bivalve collection. During 2011, Dr. G. Thomas Watters, curator of the OSUM bivalve collection, provided the updated identifications for the specimens used in this paper. In my 1983 dissertation, the concluding analysis used chromosomal characteristics to compare and contrast the relationships pro-posed in several published taxonomic group-ings of naiad genera. Present-day workers are in the midst of using genetic characteristics to reexamine relationships among the naiades. One recent publication (Graf & Cummings, 2007) proposes a new classification of this group based on an integration of morphologic, anatomical and genetic characteristics, but does not include any chromosomal charac-ters. Rather than repeat the strictly academic (and now, incomplete) analysis included in my dissertation, this paper concludes with a new analysis using chromosomal characteristics to look at relationships among species from some oft he naiad groupings proposed by Graf & Cummings (2007).

METHODS

Ideally, for chromosomal information to be used as a common and versatile tool in naiad identification and taxonomy, the preparation methods ought to meet a number of procedural and use criteria. In the field, it would be most useful if chromosome preparation techniques could provide information from specimens col-lected at any time throughout the year and be simple enough to interfere very little with other stream-side activities. In the laboratory, it would be best if chromosome preparations could provide readily retrievable, independent infor-mation about the specimens from which they were taken; be permanent, reusable records; be adaptable enough to be reanalyzed when new comparison techniques are discovered;

and, ideally, be usable on specimens already housed in a research collection.
Obviously, this set of goals is not likely to be completely met by any technique. All tech-niques require some commitment of time and expertise, and none proposed so far are usable on preserved animals. Some chromosome preparation techniques do not attempt to pro-duce permanent or widely usable slides. In fact, the aceto-orcein squash technique once used in most non-mammalian chromosome work (La Cour, 1941) produces pre-stained, temporary slides that are difficult to transport or to convert into more-permanent preparations. :

Slide Preparation Technique

At the beginning of this project, it became apparent that the most detailed chromosome comparisons then being conducted were the largely mammalian studies of various types of chromosome banding patterns. Examination of the procedures used to produce banded chro-mosomes showed that many of them involved a relatively simple basic process, produced unstained permanent slides, and appeared to be adaptable to molluscan subjects. Arrighi & Hsu (1974) describe the basic steps in this process, essentially as follows:
e if the number of mitoses is low, treat cell preparations or tissues with a metaphase arresting agent.
e treat the cell preparation with a hypotonic solution.
e fix cells in 1 glacial acetic acid: 3 methanol. e place cells on a microscope slide and either air-dry or flame-dry.
The development of a technique for naiad specimens started with these basic steps and resulted in the following procedure which, although not totally satisfactory, generally yields some chromosome spreads from most specimens:
e sever both adductor muscles at their insertion on one valve of the shell, pull the mantle loose from that valve and open the living naiad.
e remove all of the labial palp and/or part of a non-gravid gill from one side of the animal.
e cut each tissue sample into 5-10 mm pieces and place them in demineralized, double distilled water for 30 minutes.
e remove the tissue samples from the water, accept any water that will come off easily on a paper towel, then flood the samples with freshly-mixed 1 glacial acetic acid: 3 absolute methanol fixative.

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e change the fixative twice during 30-45 minutes.
e gently swab a piece of tissue over most of a labeled, clean, dry microscope slide.
e dry the slide by waving it in the air, blowing on
it, or by heating it gently high over an alcohol lamp.
The slides are permanent at this point and can be stored for staining at some later time: e for unbanded, stained chromosomes, heat the slides at 60°C for one hour, stain them in 1% Giemsa blood stain for 5-7 minutes, rinse under the tap, and allow to dry.
e mount stained slides with an oversize coverslip using Permount or some other permanent mounting medium.
The modifications from the mammalian process included in this naiad chromosome procedure were derived from a series of trial-and-error and more specific experiments on a fairly large number of naiad specimens. The bases for these modifications are discussed in the following paragraphs.

Tissue Choice: Considerable early effort was invested in determining which naiad tissue would provide the highest number of chromo-some spreads and the lowest amount of cellular or environmental debris on the slides. Tissues or structural areas examined at one time or another included: un-modified mantle, anterior free edge of the mantle, siphonal area of the mantle, labial palps, non-gravid gill, foot, gonad, kidney, and developing embryos. The edges of the mantle and the foot were apparently too firm to release many cells when swabbed onto slides. Gonadal and kidney tissues were so soft that globs of material came off on the slides and individual nuclei or chromosome spreads were obscured by this debris. Developing embryos were observed and the preparations of chromosome slides were attempted only on two occasions, both of which yielded some chromosome spreads; however, this source of chromosomal material is so rarely available that it was abandoned as a routine option.
The relative usefulness of the three remain-ing tissues (un-modified mantle, labial palps and non-gravid gill) were compared in the process of gathering chromosome spreads from a number of naiades. Un-modified mantle tissue gave some spreads; however, it often deposited a good deal of debris on the slides. Both labial palp and non-gravid gill material routinely produced clean slides but labial palp preparations almost always yielded more

chromosome spreads than non-gravid gill (average from 13 pairs of comparisons: palp — 12.8 spreads per slide, non-gravid gill — 5.0 spreads per slide).

Pretreatment. Most chromosome preparation techniques include one or more pretreatment steps to accumulate the mitotic figures (Ar-righi & Hsu, 1974; Chambers, 1982). Several experiments with Velban (vinblastine sulphate), one of the mitotic arresting agents then in common usage, failed to show any increase in spread number but did seem to cause the chromosomes to contract. A single experiment with phytohemaglutanin, a mitotic inducer, also failed to increase the number of spreads. Recent studies describe ways to improve the number of chromosome spreads on slides that were not available when the laboratory work on this project was being conducted. Cornet (1993, 1995) cultured somatic cells of the marine mus-sel Mytilus edulis in vitro for a few days and substantially increased the number of mitotic figures observed on chromosomal slides. The adaptation of this culture technique for use with freshwater bivalves would eliminate the pos-sibility of making slides in the field; however, it would improve the likelihood of gathering chromosomal data from each animal that was processed. This technique also seems to have the potential for collecting chromosomal data by taking tissue samples from — instead of sacrificing — live naiades, a change that would be especially important when working with protected species.
In another study, Woznicki & Jankun (2004) improved the mitotic index on slides from the unionid Anodonta anatina by injecting a cell proliferation stimulator (0.05 ml of 0.4% cobalt chloride) 60 hours before beginning other steps in the slide-making process. They also increased the mitotic index while reducing chro-mosomal contraction in A. anatina by quantify-ing the volume of the arresting agent that they used (from 0.5 to 1.0 ml of 0.1% colchicine solution, depending on size of the animal) before taking their tissue samples. Had any of these techniques been available in the late 1970s, they might have made a considerable difference in the results of this project.

Hypotonic Treatment: Typical hypotonic treat-ments for vertebrate animals are dilutions of 1% sodium citrate (Arrighi & Hsu, 1974). Although a number of dilutions of this material were tried, no recognizable chromosome spreads were

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observed until demineralized double distilled water was used alone as the hypotonic treat-ment. With regard to the treatment time, tissue samples of one animal were fixed after 0, 10, 20, 30, 40, 50 and 60 minutes in demineralized double distilled water. Although the number of chromosome spreads continued to increase with treatment time, the edges of individual chromosomes became increasingly fuzzy in treatments longer than 30 minutes.

Transition into Fixative: For some time after this technique should have become standard-ized, variations continued to occur in the size of interphase nuclei and, apparently, in the num-ber of chromosome figures. Careful monitoring indicated that water on the tissue samples when they were flooded with fixative reduced the size of nuclei on the resulting slides. This problem was overcome by touching the tissue samples to dry paper towels as they came out of the hypotonic treatment before placing them in a dry vial where they were flooded with the fixative.

Fixation: No substantial change was made in the composition of the fixative or the length of fixation time because experimental results indi-cated that some fixation was required but that long fixation time did not change the number of chromosome spreads observed on slides. In one case, chromosome spreads were ob-served on slides made 32 days after the tissue samples were fixed. Similar tests using tissue samples that had been in fixative for one and two years failed to produce spreads or even recognizable cell nuclei.

Slide-Making Technique: Experimentation with this aspect of the procedure was con-cerned with how to get cells on the slides and how best to dry the liquid preparations. Several tests were run “dabbing” or “swabbing” tissue samples onto dry or wet slides. The best tech-nique involved lightly rubbing the tissue sample over the surface of a dry slide, making sure to keep the sample wet with fixative. Three types of drying techniques were tested (air-drying, fanning, and warming) without determining obvious differences. The use of a standardized warming technique may help improve some staining results.

Staining (Unbanded): All staining of these chromosome preparations included the use of a stock solution of Giemsa blood stain (150 g Giemsa stain, 10 ml glycerin and 10 ml

methanol in 1 | of water). Experimental results suggested that three factors are involved in staining intensity: stain concentration, staining time, and the initial dryness oft he slides. Slides more than four days old that had been heated at 50-60°C for one hour stained well during 5-7 minutes in a 1-2% stock solution of Giemsa stain. Slides less than four days old, with or without the heat treatment, generally took stain more slowly, but Some areas were overstained. Very old slides (3 to 15 months) varied consid-erably in their acceptance of stain regardless of the one hour heat treatment; however, 5-7 minutes in 1-2% stain was most likely to yield acceptable results.

Staining (Banded): During the laboratory part of this project, a number of experiments were conducted using various vertebrate banding techniques to see if banding could be dem-onstrated on these naiad slides. Some tech-niques produced a few weakly banded naiad spreads; however, the banded chromosomes were not sufficiently clear to be considered usable, and the few banded spreads on any one slide differed from each other so much that ways to improve the staining technique were not apparent. No banded naiad chromosome spreads were photographed and studied as part of this project.

Identification and Deposition of Specimens

After the various tissue samples had been removed and processed for chromosomal use, the remaining soft parts of each specimen were preserved in 70% ethanol. Identification of each specimen was made using shell and soft part characteristics and, for nearly all specimens, was verified or corrected by Dr. David H. Stansbery, then curator of the OSUM bivalve collection. All specimens from which chromo-some slides were made were deposited and cataloged into the OSUM collection. In 2011, Dr. G. Thomas Watters, present curator of the OSUM bivalve collection, confirmed or provided updated identifications for these specimens. The cataloged specimens retain the “JJJ” numbers used during this project, as well as a label notation that chromosome slides had been prepared from them.

Location and Analysis of Chromosome Spreads

After the slides were stained, chromosome spreads were located by conducting a com-

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plete scan of the area under a 20 x 50 mm
cover slip using a 200x lens combination on
an Olympus phase contrast microscope. Well-separated spreads that included indications of centromeres or arms were photographed us-
ing a Pentax Spotomatic camera mounted on the Olympus phase contrast microscope with a built-in light source. All photographs were taken on Kodak High Contrast Copy Film at 1500x, at between one and four seconds for well-stained spreads, or at 15 or 30 seconds for lightly-stained spreads enhanced with a green filter. All usable negatives were printed to the same scale to simplify comparisons between spreads. Individual chromosomes on the photographs were outlined and, when possible, the centromeres were located while comparing the photograph to the ac-tual spread magnified to 1500x through the microscope.
Digital calipers were used to measure the outlined chromosomes on the photographs. Generally, the longer arm on each end of the chromosome was measured from the cen-tromere to the tip. When the chromatid arm or the centromere could not be distinguished, the greatest length of the arm or of the entire chromosome was measured. All measure-ments for arms or entire chromosomes were recorded at the nearest 0.05 mm. Two metrics were calculated for each chromosome: Arm Ratio (r) and Relative Length (RL).

FIG. 1. A good quality spread of the 38 chromo-somes from the labial palp of the North American naiad Villosa trabalis.

RESULTS AND DISCUSSION

During this project, 250 naiades representing approximately 75 species were used as sourc-es of chromosomal material. These animals were collected between August 1975, and May 1978, from ten of the east-central and south-eastern United States, the western state of Oregon, and Manitoba Province, Canada. They yielded chromosome counts for 65 species in 34 presently-recognized naiad genera. Overall, of the 771 spreads that were counted, 502 (65%) included 38 chromosomes (Fig. 1). Five or more chromosome counts were obtained from 42 of these species, and 38 was the clear modal number for each of them. Two-hundred-five superior quality chromosome spreads were photographed, 125 of them (representing 39 species in 24 genera) were measured, and the 87 measured spreads that included exactly 38 chromosomes were analyzed. Table 2 presents a species-by-species summary of the chromo-some counts and analysis results that were generated during this project.

Chromosome Number

The information presented in Table 2 indi-cates that 38 is the modal number of diploid chromosomes found during this study of North American naiades. Figure 2 reinforces this assertion by tallying the numbers of chromo-somes observed in all of the 771 countable spreads encountered during this project. The clear mode of these counts at 38 (65% of the total), accompanied by the sharply skewed distribution pattern, strongly suggests that 38 is the typical chromosome number present in these naiades. The 257 counts that were lower than 38 (33% of the total) most likely consist of spreads where some chromosomes landed on top of others or where chromosomes were washed away when the cell membranes rup-tured during slide preparation. The 14 counts higher than 38 (2%) probably include some separated chromatids, the results of atypical cell divisions, and — more much more rarely on these slides — chromosomes that had floated in from other spreads.
On an individual basis, each of the 42 spe-cies represented by five or more counts had a modal number of 38 chromosomes (Table 2). In addition, among the 23 species represented by between one and four counts, the strongest

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Number of Chromosomes

FIG. 2. The numbers of naiad chromosomes observed in the 773 spreads counted during this project. The 502 counts with the modal number (38) make up 64.9% of the total.

suggestion of a modal number other than 38
was for Quadrula infucata (four counts: 31-1,
36-2, 38-1). These results reinforce the con-
clusion that 38 is the diploid chromosome num-
ber for all 65 naiad species that were studied,
including Quadrula infucata.
The conclusion that all 65 North American naiad species examined during this study have 38 chromosomes adds substantial new information to the compilation of published naiad chromosomal numbers presented in Table 1. Considered together, Tables 1 and 2 indicate that the 67 species of North American naiades that have been examined [15 reported in Jenkinson (1976), two added by Park & Burch (1995) and 50 first reported here] all have 38 chromosomes. These North American species are considered members of two naiad families: Unionidae and Margaritiferidae (Graf & Cummings, 2007). Similarly, all 11 naiad species studied in Europe (all in Unionidae) and 14 ofthe 15 naiades studied in Asia (both

Margaritiferidae and Unionidae) also have 38 chromosomes. [The 15th Asian species, Sole-nia khwaegnoiensis, was reported to have 37 diploid chromosomes; however, the haploid number was still listed as 19 (Deein et al., 2003).] The one unionid species studied so far in Africa (Unio elongatulus) is reported to have 28 chromosomes (Ebied, 1998); however, an Italian study of what was identified as the same species is reported to have 38 chromosomes (Vitturi et al., 1982). With the possible excep-tions of Solenia khwaegnoiensis and African Unio elongatulus, 38 is the diploid chromo-some number known for all of the unionid and margaritiferid species that have been studied. All of these species occur in the Northern Hemisphere, and all of them are considered members of the superfamily Unionoidea (Graf & Cummings, 2007).
The five remaining naiad species listed in Table 1 are all considered members of the superfamily Etherioidea (Graf & Cummings,

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2007). Four ofthese species (Alathyria pertex-ta, Diplodon chilensis, Velesunio ambiguus and Velesunio legrandi — all in the family Hyriidae) have 34 chromosomes, and the fifth (Mutela rostrata in the family Iridinidae) has 20 chro-mosomes. As indicated in Table 1, three о the hyriid species occur in Australia and Tazmania, while the fourth (Diplodon chilensis) is found in Chile. The iridinid species occurs in Egypt. Obviously, the chromosome numbers of these five mostly Southern Hemisphere etherioid spe-cies are different from those ofthe much larger number of Northern Hemisphere unionoids. In their broad-spectrum cladistic analysis of relationships within the bivalve subclass Palaeoheterodonta, Graf & Cummings (2006) conclude that the order Unionoida is mono-phyletic and can be represented by eight morphologic synapomorphies: four characters associated with gill morphology, and four as-sociated with life history and reproductive characters. Their analysis also indicates this order can be divided into two superfamilies (Etherioidea and Unionoidea); however, few morphological characters align with the distinctions indicated by the DNA data. The chromosome numbers reported so far for the naiades suggest a clear distinction between the 38 present in both the Margaritiferidae and the Unionidae of the Northern Hemisphere (the Unionoidea) and different numbers (34 for the Hyriidae and 20 for at least one iridinid from Africa) in the Etherioidea of the Southern Hemisphere. If this difference in chromosome numbers is substantiated as more, especially Southern Hemisphere naiades are studied, this straight-forward morphologic character could become important in recognizing distinctions between high-level groups of naiades.

Supernumerary Chromosomes

The chromosome slides from three animals studied during this project include a chromo-somal feature not indicated in Table 2. One slide each of Leptodea fragilis, Obovaria olivaria, and Pyganodon g. grandis contained a few spreads that included a small “dot” chromosome. Single dots were present in six of 14 spreads (43%) noted on the P g. grandis slide, four of 21 spreads (19%) on the L. fragilis slide, and one of 14 spreads (7%) on the O. olivaria slide. The dot in two measured Р д. grandis spreads comprised 0.64 and 0.81% of the total complement length, less than half the length of the next smallest chromosome

(Fig. 3). No centromere was observed on any of the dots.
The dot chromosomes on these па!аа slides appear to fit the general description of super-numerary ог “В” chromosomes (Jones & Hou-ben, 2003; Palestis et al., 2010). As a group, supernumeraries are small chromosomes that occur infrequently in species and individuals, and appear to act essentially as chromosomal parasites. Supernumerary chromosomes have been found in fungi, plants and animals; may or may not contain active genetic material that can affect phenotypes; may deviate from nor-mal duplication and separation patterns during cell division (nondisjunction); and may occur in a variety of numbers within the cells of an organism (Camacho et al., 2000).
Patterson (1969) cited a few cases of super-numerary chromosomes in gastropods but none in other mollusks. Some ofthe cases, Patterson presented appear to be similar to the rare oc-currence, non-uniform pattern found during this project. Other cases, however, seem more likely to have been various types of disruptions of the normal chromosome complement. The first published description of supernumerary chromosomes in bivalves was in a report by Insua & Thiriot-Quievreux (1992). Leitäo et al. (2008) used banding techniques to identify the supernumeraries they found in the marine bivalve Cerastoderma edule as fragments of the normal chromosomes and suggested that fissions in the normal chromosomes might have been caused by environmental pollution.

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FIG. 3. À chromosome spread from the labial palp of Pyganodon grandis grandis including 38 normal chromosomes and (at arrow) one supernumerary chromosome.

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Chromosome Morphology

The number of chromosomes is not the
only chromosomal characteristic that might
be useful in identifying relationships among
naiad species, genera, and higher taxa. In
many animal and plant groups, variations in the relative lengths of the chromosomes and in the location of the centromere on each chromosome - the karyotype of an individual, population, or species — provide quantifiable features that can be analyzed and compared. In recent years, karyotypic analyses have be-come much more common than simple lists of chromosome numbers and are supplying more useful data than was previously thought avail-able from chromosomal work (Takagi & Sasaki, 1974; Bogart, 1973; Melnychenko & Yanovich, 2004). Karyotypic studies on mollusks were begun by Burch (1962) and Patterson (1965), and continue to increase in sophistication and application (e.g., Babrakzai et al., 1976; Naka-mura, 1985; Thiriot-Quievreux, 2002).
In most analyses, the chromosomes within a spread are sorted into groups based on the position ofthe centromere. Typically, the length of the longer arm of a chromosome is divided by the length of the shorter arm and the result is presented as the Arm Ratio (r) (Thiriot-Quiévreux, 2002).
Another useful chromosomal metric, Relative Length (RL), is calculated by dividing the length of each chromosome (long arm plus short arm) by one-half the length of all the chromosomes inthe spread, then multiplying the result by 100 to convert it into a percentage of the haploid length (Petkeviciüte et al., 2007). Аррагепйу, RL is nearly always presented with reference to the haploid length because this metric was first used in regard to human chromosomes and, in order to avoid differences in RL values between the sexes, the female haploid (auto-some) complement was used as its basis (Chu & Giles, 1959). |
The г and RL metrics are virtually indepen-dent of each other. The r value describes the relative location of the centromere without re-gard to the overall length of any chromosome. The RL value compares the total length of one chromosome to the aggregate length of all chromosomes in the spread without regard to the location of any centromere. These metrics are virtually independent of each other because obscured folding along the length of a chromo-some and other measurement errors will affect the values of both r and RL.

It is also important to realize that both r and RL are continuous variables. The stabilization in Arm Ratio terminology proposed by Levan et al. (1964) includes a clear description of the range of possible locations of the centromere (from 1.00 to well above 7.00), along with the authors’ logic for the mathematical break points between the metacentric (m; r = 1.00—1.70), submeta-centric (sm; г = 1.71-3.00), subtelocentric (st; г = 3.01-7.0) and acrocentric [= telocentric] (t; r > 7.00) segments they established.
No comparable separation of RL values into consistent segments is likely to be widely applicable because of the wide range in chro-mosome numbers (and, therefore, the relative lengths of the individual chromosomes) among animal and plant species. Early in this project, the break points between segments of the RL spectrum were arbitrarily set at 2% intervals of the haploid length; however, so few data points fell below 2% or above 10% that the outlying intervals were combined to produce the 0-4.0%, 4.01-6.0%, 6.01-8.0%, and > 8.0% RL segments used in this analysis.
With regard to the r metric, most the chromo-somes in the 87 measured 38-chromosome spreads included in this study have centrom-eres located near the center (53% are metacen-tric), followed in abundance by submetacentrics (36%), subtelocentrics (11%) and telocentrics (0.2%). The virtual absence of telocentric chro-mosomes indicates there is little or no value in comparing “fundamental numbers” among these karyotypes. The concept of counting all of the arms of the chromosomes in a spread was proposed by Matthey (1949) and has been used to describe situations in which several single-armed (telocentric) chromosomes ex-ist in some species while fewer telocentrics occur in others. This absence of telocentrics, when combined with the uniform 38 diploid chromosome number present in these species produces a fundamental number of 76 for all of the karyotypes analyzed during this study. With regard to the RL data, most of these chromosomes (67%) occur in the 4-6% range, about the average length expected for spreads including 38 chromosomes (100%/19 haploid chromosomes = 5.3 RL). Approximately 11% (in the 0-4% range) are shorter than average, 19% (in the 6-8 RL range) are slightly longer than average, and 3% (in the > 8 RLrange) are substantially longer than average. Many recent karyotypic papers include RL values as part of the description of the chromosomes being ex-amined (e.g., Pasantes et al., 1990; Insua et al.,

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1994; Gonzälez-Tizön et al., 2000; Iqbal et al., 2008); however, | could not find any previous study that has sorted the RL data into segments and used it as an analytical tool.

Measurement Error

The comparisons of naiad karyotypes pre-sented below are based solely on measure-ment data and the analyses of the r and RL metrics assume that whatever errors might be included in the measurements were not large enough to materially affect the results. The validation of this assumption requires an examination of measurement error.
A review ofthe measurement data indicated that naiad species within the genus Villosa have two pairs of distinctive subtelocentric chromosomes, typically in the 4-6 RL size range that could be studied to determine the margin of error in their measurement. The four chromosomes with the largest r values in each of the 22 Villosa spreads available for this comparison were sorted into pairs based solely on their RL values. Mean and standard deviation values were calculated for the short arms, long arms, and for the full lengths of each chromosome pair (Table 3). If the means of these RL measurements are taken to represent the actual (relative) lengths of the chromosome segments, and the stan-dard deviations are taken to estimate the total effects of inconsistencies in outlining, locating the centromeres, and measuring them, then the Coefficient of Variation (100s/x) based on all of the pairs included in each test is an estimate

TABLE 3. Relative lengths of two pairs of subte-locentric chromosomes in 22 measured spreads from four species in the genus Villosa. The Coefficient of Variation (CV) was calculated by dividing 100 times the Standard Deviation (SD) by the Mean.

Segment n Mean SD CV

Longer RL Pair
Short Arms 44 403 : 10:63" 15.63
Long Arms 44 15:39 0 10,97 6.33
Total Length 44 19.42 1.33 6.85

Shorter RL Pair
Short Arms 44 3.32 -- 0,45. 13,40
Long Arms 44 12.83 -11:07 8.32
Total Length 44 TOS . 11.20 7.93

of the error in these measurements (Steel & Torrie, 1960). The data presented in Table 3 indicate that error was greatest (15 and 13%) for the short arms of these two chromosome pairs and approximately half of that (6 to 8%) for the long arms and for the total lengths. The average error value based on the four arm measurement tests was 10.9%.
Future projects involving the measurement of naiad chromosomes are likely to avoid at least this high a level of measurement error. Recent chromosomal studies, such as Martinez-Lage et al. (1996) and Skuza et al. (2009), cover this aspect of the work simply by identifying the computer programs they ran to take the measurements.

Karyotype Analysis

As indicated in Table 2, 125 high quality chromosome spreads (representing 39 species in 24 genera) were measured and analyzed during this project. Unfortunately, 38 of these spreads had fewer (29 spreads, 27%) or more (9 spreads, 3%) than exactly 38 chromosomes. With the exclusion of these “imperfect” spreads, the following analysis includes 87 38-chromo-some spreads representing 31 species in 21 genera.
Because r and RL are virtually independent metrics, analyzing them together makes it possible to sort the sizes and shapes of chro-mosomes from a spread or a species in a more detailed way than is possible by examining either metric alone. Sorting the number of chromosomes that plot in each of the four r seg-ments into whichever of the four RL segments they also represent divides the data into as many as 16 unique size/shape categories. Eighteen species included in this study were each represented by two or more measured 38-chromosome spreads. For this analysis, the r and RL metric data from the spreads of each species were averaged together to identify the most likely even (diploid) number of chromosomes within each of the 16 possible r/RL categories that would add up to the most likely rand RL segment totals and to the overall total of 38 chromosomes. Both the r and RL segment values of the suggested karyotypes for these 18 species are presented in Table 2. Following the approach typically used for Arm Ratio karyotypes (e.g., Nakamura, 1985; Leitao et al., 1999a) and for Relative Lengths when they are listed (e.g., Park & Burch, 1995; Car-rilho et al., 2008), the suggested karyotypes in Table 2 are presented using haploid numbers.

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Tables 4 and 5 present some of the results of
this analysis. In both of these tables, the four r metric segments form the rows, and the four RL segments form the columns. The totals for the
r segments are presented along the right side
and the RL segment totals are presented along the bottom. In Table 4, the upper left number in each cell and for each segment total is the most likely even (diploid) number of chromosomes that plot within both that r and RL segment based on the eight 38-chromosome spreads of Villosa trabalis that were measured during this study. The lower right value in each cell and total is the comparable chromosome number based on the seven 38-chromosome spreads of Villosa taeniata punctata that were included. These two species are typically placed in the same naiad genus (Parmalee & Bogan, 1998; Graf & Cummings, 2007).
As indicated in the right-hand column of Table 4, the suggested r karyotypes of these two species are identical to each other; however, the bottom row indicates that their suggested RL karyotypes differ in three of the four RL segments. The detailed karyotypes (16 inte-rior table cells) indicate that V. t. punctata has four chromosomes (two pairs) more than V. trabalis that plot in the т(0—4) cell and two fewer chromosomes (one pair) that plot in both the m(4-6) and m(6-8) cells. The most

TABLE 4. Comparison of the detailed karyotypes of Villosa trabalis (upper left numbers in each cell) and Villosa taeniata punctata (lower right numbers). These two species have identical sug-gested Arm Ratio karyotypes (right column totals) but they differ in 3 of the 4 suggested Relative Length karyotype categories (bottom row totals) and in three 3 of the 16 detailed karyotype cells [m(0-4), m(4-6), and m(6-8)].

Relative Length (RL)

R(Arar)tm i o

Totals

parsimonious set of chromosomal modifica-tions that could produce this difference in these karyotypes would involve the deletion (or, conversely, the duplication) of part of the lengths of two different chromosome pairs (two separate chromosomal modifications), neither of which was sufficient to move them from the metacentric to the submetacentric segment of the r metric. In general, changes in RL values indicate additions or deletions in overall chro-mosome length (duplications, deletions, etc.), while changes in r values indicate relocations in the position of the centromeres (inversions, translocations, etc.).
Table 5 presents a similar comparison between Villosa trabalis (upper left, based on eight measured spreads) and Lasmigona costata (lower right, based on six measured spreads), two species that are generally con-sidered to be in different naiad subfamilies or tribes (Vokes, 1980; Graf & Cummings, 2007). The suggested r (right column totals) and RL (bottom row totals) karyotypes for these spe-cies differ from each other in six of the eight metric segments (all but telocentrics and 6-8 RL). The detailed karyotypes of these species differ from each other in all eight of the cells

TABLE 5. Comparison of the detailed karyotypes of Villosa trabalis (upper left numbers in each cell) and Lasmigona costata (lower right numbers). The suggested Arm Ratio (right column totals) and Relative Length (bottom row totals) karyotypes for these species differ from each other in 6 of the 8 categories (all but telocentrics and RL 6-8). Similarly, the detailed karyotypes of these species differ from each other in all 8 of the cells where naiad chromosomes usually plot [m(0—4), m(4-6), m(6-8), m(> 8), sm(0-4), sm(4-6), sm(6-8), and st(4-6)].

Relative Length (RL)

R(Arar)tm i o

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where these naiad chromosomes usually plot [m(0-4), m(4-6), m(6-8), m(> 8), sm(0-4), sm(4-6), sm(6-8), and st(4-6)]. Assuming that each 2-chromosome difference (either increases or decreases, but not both) in the de-tailed karyotypes represent one chromosomal modification, Villosa trabalis and Lasmigona costata differ from each other by at least five separate duplications, deletions, translocations or other changes in the morphology of their chromosomes. This number of differences is large enough that several possible sets of chromosomal modifications are equally likely to have produced the detailed karyotypes that were observed.
Table 6 expands this species-by-species comparison to include the ten species that are each represented by four or more measured 38-chromosome spreads in addition to a com-posite karyotype derived from all 87 measured 38-chromosome spreads included in this analy-sis. The third column in Table 6 indicates the number of cells in which the detailed karyotype value for each species differs from the com-posite, and the succeeding columns indicate the number of cells in which pairs of species have either more or fewer chromosomes in a

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cell than the composite. Highlighted values in Table 6 indicate sets of species that share pairs of cells in which their detailed karyotype values differ from the composite in complementary ways (one cell with +2 and the other with -2 chromosomes different from the composite). Single values in the species-by-species part of Table 6 are assumed to represent random occurrences of the same difference from the composite. Larger numbers of occurrences that are not highlighted represent situations in which both species differ from the composite in the same way in those cells (both species are either higher or lower than the composite in both cells) or where one of the occurrences is committed to a different, multi-species relationship.
The relationships identified in Table 6 are illustrated on Figure 4. The concentric circles on this figure represent the number of chromo-somal modifications by which the species and the composite differ from each other. Straight lines indicate the identified (solid) and alterna-
tive (dashed) connections between species
and the composite. For example, Ligumia
nasuta and Ptychobranchus fasciolaris share
one pair of differences from the composite [an
inversion that relocates a pair of chromosomes

TABLE 6. Apparent relationships among the detailed karyotypes of the 10 naiad species and the 87-spread composite included in this analysis. The species are arranged here in descending order based on the number of measured spreads they represent. The meaningful relationships (highlighted cells) are illustrated on Figure 4.

Number of Shared Differences from Composite

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Villosa trabalis 8 2 ы
Villosa t. punctata F4 4 1
Lasmignona costata 6 8 0
Obovaria subrotunda 6 4 1
Pyganodon g. grandis 6 2 0
Villosa iris 6 2 1
Actinonaias I. carinata À 4 1
Ligumia nasuta 4 4 0
Pleurobema rubrum À 10 2
Ptychobranchus fasciolaris 4 6 0

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FIG. 4. Apparent relationships among the detailed karyotypes of 10 naiad spe-cies and a composite based on all 87 38-chromosome spreads included in this analysis. Each ring on this figure represents one chromosomal modification from the adjacent ring or the center. Abbreviations: Ac — Actinonaias |. carinata, С — Composite of all 87 spreads, Lc — Lasmigona costata, Ln — Ligumia nasuta, Os — Obovaria subrotunda, Pf — Ptychobranchus fasciolaris, Pg — Pyganodon g. grandis, Pr — Pleurobma rubrum, Vi — Villosa iris, Vp — Villosa taenaita punctata, and Vt - Villosa trabalis.

from m(4-6) to sm(4-6)], then Ligumia nasuta exhibits one more modification, while Ptycho-branchus fasciolaris exhibits two different modi-fications away from the composite. Similarly, Obovaria subrotunda, Actinonaias ligamentina carinata and Villosa taeniata punctata all share one modification from the composite [a deletion that relocates one pair of chromosomes from sm(4-6) to m(0-4)], then each of them exhibits an additional, different modification from the composite.
As indicated in Table 6 and illustrated on Figure 4, Pleurobema rubrum differs from the composite by five chromosomal modifications. One of those modifications also occurs in Vil-losa trabalis while another occurs in Villosa iris; however, those two Villosa species do not share any difference from the composite. As a result, Figure 4 indicates that either Villosa trab-alis or Villosa iris could be part of the sequence of modifications separating the karyotype of Pleurobema rubrum from the composite.
The overall image conveyed by Figure 4 is that most of these ten species form a fairly tight cluster around the center (the composite), while

two species are clear outliers. Examination of Table 2 indicates that most of the 87 spreads that contributed to the composite (56 spreads, 64% of the total) are from species typically considered in the subfamily or tribe Lampsilini (Vokes, 1980; Graf &C ummings, 2007). Given the large number of lampsiline spreads in-cluded in the composite, it is not surprising that most of the seven lampsiline species included in this analysis plot near the center on Figure 4, and that the single pleurobemine species (Pleurobema rubrum) and one of the two ano-dontine species (Lasmigona costata) plot so far from the center. What may be more interesting is the fact that Pyganodon g. grandis, typically considered an anodontine species (Vokes, 1980; Graf & Cummings, 2007), plots just one chromosomal modification away from this lampsiline-dominated composite. This figure also suggests that Ptychobranchus fasciolaris has a more divergent karyotype than the other lampsiline species included in this analysis. While the possible karyotypic relationships presented in Figure 4 may be interesting to some, the long-term value of this analysis is

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more likely to be associated with the process in-stead ofthese specific results. The ten species included in this analysis are each represented by between four and eight measured chromo-some spreads and, as indicated above, the mar-gin of error associated with the measurement ofthese chromosomes is about 11%. In spite of its small sample sizes and relatively large error component, this analysis does associate most of the species thought to be members of the same naiad tribe, and does segregate out two of the three species thought to be members of different tribes. In other words, even this small and relatively noisy analysis of these two chro-mosomal metrics, when considered together, is able to demonstrate similarities and differences among naiad species that correspond with results from other sets of morphological and genetic characteristics. This conclusion sug-gests that the potential benefits of exploring this entirely new set of naiad morphological charac-teristics is likely to justify the effort necessary to improve the quality of the chromosomal mea-surements, increase the number of measured spreads per species, and augment the number of species that can be included.

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